(54) COUPLAGE DE PROTEINES AVEC UN POLYSACCHARIDE MODIFIE
(54) COUPLING PROTEINS TO A MODIFIED POLYSACCHARIDE

(57)
The invention relates to a method for coupling proteins to a starch-derived modified polysaccharide. The binding interaction between the modified polysaccharide and the protein is based on a covalent bond which is the result of a coupling reaction between the terminal aldehyde group or a functional group of the modified polysaccharide molecule resulting from the chemical reaction of this aldehyde group and a functional group of the protein which reacts with the aldehyde group or with the resulting functional group of the polysaccharide molecule. The bond directly resulting from the coupling reaction can be optionally modified by a further reaction to the aforementioned covalent bond. The invention further relates to pharmaceutical compositions that comprise conjugates formed in this coupling process and to the use of said conjugates and compositions for the prophylaxis or therapy of the human or animal body.

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(54) Titre : COUPLAGE DE PROTEINES AVEC UN POLYSACCHARIDE MODIFIE
(54) Title: COUPLING PROTEINS TO A MODIFIED POLYSACCHARIDE

(57) Abrégé/Abstract:
The invention relates to a method for coupling proteins to a starch-derived modified polysaccharide. The binding interaction between the modified polysaccharide and the protein is based on a covalent bond which is the result of a coupling reaction between the terminal aldehyde group or a functional group of the modified polysaccharide molecule resulting from the chemical reaction of this aldehyde group and a functional group of the protein which reacts with the aldehyde group or with the resulting functional group of the polysaccharide molecule. The bond directly resulting from the coupling reaction can be optionally modified by a further reaction to the aforementioned covalent bond. The invention further relates to pharmaceutical compositions that comprise conjugates formed in this coupling process and to the use of said conjugates and compositions for the prophylaxis or therapy of the human or animal body.

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(54) Title: COUPLING PROTEINS TO A MODIFIED POLYSACCHARIDE

(54) Bezeichnung: KOPPLUNG VON PROTEINEN AN EIN MODIFIZIERTES POLYSACCHARID

(57) Abstract: The invention relates to a method for coupling proteins to a starch-derived modified polysaccharide. The binding interaction between the modified polysaccharide and the protein is based on a covalent bond which is the result of a coupling reaction between the terminal aldehyde group or a functional group of the modified polysaccharide molecule resulting from the chemical reaction of this aldehyde group and a functional group of the protein which reacts with the aldehyde group or with the resulting functional group of the polysaccharide molecule. The bond directly resulting from the coupling reaction can be optionally modified by a further reaction to the aforementioned covalent bond. The invention further relates to pharmaceutical compositions that comprise conjugates formed in this coupling process and to the use of said conjugates and compositions for the prophylaxis or therapy of the human or animal body.

(57) Zusammenfassung: Die vorliegende Erfindung betrifft die Kopplung von Proteinen an ein von Stärke abgeleitetes modifiziertes Polysaccharid, wobei die bindende Wechselwirkung zwischen dem modifizierten Polysaccharid und dem Protein auf einer kovalenten Bindung beruht, welche das Ergebnis einer Kopplungsreaktion zwischen der endständigen Aldehydgruppe oder einer aus dieser Aldehydgruppe durch chemische Umsetzung hervorgegangenen funktionellen Gruppe des modifizierten Polysaccharidmoleküls und einer mit dieser Aldehydgruppe oder daraus hervorgegangenen funktionellen Gruppe des Polysaccharidmoleküls reaktionsfähigen funktionellen Gruppe des Proteins ist, wobei die bei der Kopplungsreaktion unmittelbar resultierende Bindung gegebenenfalls durch eine weitere Reaktion zur obengenannten kovalenten Bindung modifiziert sein kann. Die Erfindung betrifft ferner pharmazeutische Zusammensetzungen, welche die bei der Kopplung gebildeten Konjugate umfassen, und die Verwendung dieser Konjugate und Zusammensetzungen zur prophylaktischen oder therapeutischen Behandlung des menschlichen oder tierischen Körpers.

Coupling proteins to a modified polysaccharide

The rapid development in genetic engineering in recent
decades has led to the new identification of a large number
5 of genes for proteins having potential therapeutic benefits
and to the possibility of producing without difficulty the
corresponding gene products, pure or nearly pure in
relatively large quantities, with the aid of biological
expression systems.
10

However, it has emerged that the use of such proteins in
practice, e.g. in diagnosis, therapy and for
biotransformations, frequently meets with difficulties
because the stability and solubility properties thereof,
15 especially at physiological pH values, are often
unsatisfactory. Two examples of such proteins are tumor
necrosis factor TNF-α or interleukin-2.

Solubility problems additionally occur very frequently in
20 the expression of glycoproteins in prokaryotic systems such
as E. coli, because they are then expressed without the
natural glycosylation, resulting in a considerably reduced
solubility in some cases. This may make it necessary to use
considerably more costly eukaryotic expression systems.
25

On therapeutic use in the body, many proteins are very
quickly removed from the bloodstream or degraded.
Systemically administered proteins having a molecular
weight of more than about 70 kD may be removed from the
30 circulation by the reticuloendothelial system or specific
interactions with cellular receptors. Smaller proteins
having a molecular weight of less than about 70 kD may in
addition be removed to a large extent by the glomerular
filtration in the kidney (exclusion limit about 70 kD).

An approach followed recently to eliminate the described problems consists in coupling such problematic proteins to biocompatible polymers with good solubility in water, such as, for example, polyethylene glycol and dextran. On the one hand, it is possible by the coupling to increase the molecular weight above the threshold of 70 kD, so that the plasma residence time of smaller proteins can be drastically increased, and on the other hand the solubility in aqueous medium can be improved by the hydrophilic polymer portion.

Further, usually beneficial effects which may be connected with coupling of proteins to such polymers are based on the masking of protease recognition sites and antigenic determinants on the protein molecule by the bound polymer. On the one hand, it is possible thereby for the therapeutic proteins substantially to escape proteolytic degradation, and on the other hand there is substantial suppression of the induction of allergenic reactions by the exogenous therapeutic protein. Beyond the increase in molecular weight, proteins are thus protected by the presence of a polymer from enzymatic degradation and, in addition, often from thermal denaturation. In many cases, the stability and in vivo half-life of the proteins is markedly increased, and the immunogenicity and antigenicity falls, thereby.

To date, most modifications have been carried out with polyethylene glycol or dextran, with PEG being generally preferred because it affords simpler products.

Dextran couplings have been described for only a few proteins such as, for example, streptokinase, plasmin, hemoglobin or aprotinin. However, dextran conjugates often show high allergenicity, presumably caused by dextran degradation products, a low metabolic stability and, in many cases, low yields in the coupling reactions. This has

led to none of these dextran coupling products being approved as yet for therapeutic use in humans or animals.

Derivatizations with PEG have been carried out considerably
5   more frequently, so that this method can now be regarded as standard for increasing the molecular weight of proteins. Some of these derivatives are in various phases of clinical trials or are already approved in the USA. PEG-hemoglobin is currently in phase III, as is a PEG adduct of superoxide
10 dismutase (SOD), which is the protein which has been investigated most in relation to polymer couplings. PEG-coupled asparaginase is already employed in the therapy of acute lymphocytic leukemia. In 2001, PEG-interferon-α was approved for the treatment of hepatitis C patients.
15

On use of these PEG conjugates, however, side effects ranging from unpleasant to dangerous have also been reported, such as pruritis, hypersensitivity reactions and pancreatitis. In addition, the biological activity of the
20 proteins after PEG coupling is often very low and the metabolism of the degradation products of PEG conjugates is still substantially unknown and possibly represents a health risk.

25 WO 99/49897 describes conjugates of hemoglobin which are formed by reacting the aldehyde groups of oxidatively ring-opened polysaccharides such as hydroxyethylstarch or dextran with primary amine groups of the protein. However, in this case, the employed polysaccharides act as
30 polyfunctional reagents, resulting in a very heterogeneous product mixture with properties which are difficult to adjust.

US patent 6,083,909 describes a process for coupling
35 selectively oxidized hydroxyethylstarch to hemoglobin in DMSO. Our investigations have shown, however, that the

desired product is not obtained under the stated conditions, because hemoglobin is denatured in DMSO and thus loses its biological activity.

5   There is thus still a need for physiologically well tolerated alternatives to dextran- or PEG-coupled proteins, with which the solubility of proteins can be improved or the plasma residence time of the proteins can be increased.

10   It is therefore an object of the invention to provide such alternatives and to develop simple and efficient processes for preparing such alternative protein derivatives.

This object is achieved according to the invention by
15 hydroxyalkylstarch-protein conjugates which are characterized in that the binding interaction between the hydroxyalkylstarch molecule and the protein is based on a covalent bonding which is the result of a coupling reaction between the terminal aldehyde group, or a functional group
20 derived from this aldehyde group by chemical reaction, of the hydroxyalkylstarch molecule and a functional group, which is able to react with this aldehyde group or functional group derived therefrom of the hydroxyalkylstarch molecule, of the protein, where the
25 bonding resulting directly in the coupling reaction can be modified where appropriate by a further reaction to give the abovementioned covalent bonding.

The invention further includes pharmaceutical compositions
30 which comprise these conjugates, and the use of these conjugates and compositions for the prophylactic or therapeutic treatment of the human or animal body, and methods for preparing these conjugates and compositions.

35   It has surprisingly been found that the reactions described above can, with a suitable choice of the conditions, be

carried out in aqueous solution, thus allowing the biological activity of the proteins in many cases to be completely or partly retained.

5   The aqueous reaction medium for the coupling reaction is in this case preferably water or a mixture of water and an organic solvent, where the proportion of water in the mixture is at least about 70% by weight, preferably at least about 80% by weight, more preferably at least about
10   90% by weight.

The molar ratio of hydroxyalkylstarch (HAS) to protein in the coupling reaction is usually about 20:1 to 1:1, preferably about 5:1 to 1:1.

15

The remaining biological activity of the inventive hydroxyalkylstarch-protein conjugates, based on the initial activity of the protein, is usually at least 40%, preferably at least 50%, more preferably at least 70%, even
20   more preferably at least 90%, most preferably at least 95%.

The hydroxyalkylstarch (HAS) employed according to the invention can be prepared by a known method, e.g. hydroxyalkylation of starch at the C_2 and/or C_6 position of
25   the anhydroglucose units with alkylene oxide or 2-chloroalkanol, e.g. 2-chloroethanol (see, for example, US 5,218,108 for the hydroxyethylation of starch), with various desired molecular weight ranges and degrees of substitution. It is also possible to employ any
30   preparations obtainable commercially. The definition of the alkyl grouping in "hydroxyalkylstarch", as used herein, includes methyl, ethyl, isopropyl and n-propyl, with particular preference for ethyl. A substantial advantage of HES is that it is already approved by the authorities as
35   biocompatible plasma expander and is employed clinically on a large scale.

The average molecular weight of the hydroxyalkylstarch can be in the range from about 3 kD to several million daltons, preferably about 4 kD to about 1000 kD, more preferably in the range from about 4 kD to about 50 kD or in the range from about 70 kD to about 1000 kD, particularly preferably about 130 kD. For coupling to small proteins, the average molecular weight of the hydroxyalkylstarch is preferably chosen so that the abovementioned threshold of 70 kD is exceeded with the conjugates, whereas for coupling to large proteins the molecular weight of the hydroxyalkylstarch will preferably be in the lower region of said range. Since coupling is possible at a plurality of sites in a protein, it may also be advantageous to couple a plurality of small polymer chains, instead of one of high molecular weight. The degree of substitution (ratio of the number of modified anhydroglucose units to the number of anhydroglucose units in total) may likewise vary and will frequently be in the range from about 0.2 to 0.6, preferably about 0.3 to 0.7, more preferably about 0.5. (Note: the numbers relate to the "degree of substitution", which is between 0 and 1). The ratio of C_2 to C_6 substitution is normally in the range from 4 to 16, preferably in the range from 8 to 12.

These parameters can be adjusted by known methods. Experience with the use of hydroxyethylstarch (HES) as blood substitute has shown that the residence time of HES in the plasma depends on the molecular weight and the degree of substitution and type of substitution (C_2 substitution or C_6 substitution), with a higher molecular weight, a higher degree of substitution and a higher proportion of C_2 substitution increasing the residence time.

These relationships also apply to the inventive hydroxyalkylstarch-protein conjugates, so that the

residence time of a particular conjugate in the plasma can
be adjusted via the proportion of polysaccharide.

Hydroxyethylstarch products with an average molecular
5 weight of 130 kD and a degree of substitution of 0.5, and
with an average molecular weight of 200 kD and a degree of
substitution of 0.25, have already been used clinically as
blood substitutes and are also suitable for use in the
present invention.
10

The protein suitable in the present invention is in
principle any protein which has the necessary functional
group, e.g. a free amino group, thiol group or carboxyl
group, for reacting with the functional group of the HAS
15 molecule.

A desired functional group can be introduced also by
reacting the protein with a suitable, physiologically
tolerated, bifunctional linker molecule. The remaining
20 reactive functional group of the coupled-on linker molecule
is then likewise regarded as "reactive functional group of
the protein" for the purposes of the present invention.

Suitable linker molecules comprise at one end a grouping
25 able to enter into a covalent bonding with a reactive
functional group of the protein, e.g. an amino, thiol, or
carboxyl group, and at the other end a grouping likewise
able to enter into a covalent bonding with the terminal
aldehyde group or a functional group derived therefrom by
30 chemical reaction, e.g. a carboxyl group, activated
carboxyl group, amino or thiol group. Between the two
functional groups of the linker molecule there is a
biocompatible bridging molecule of suitable length, e.g. a
grouping derived from an alkane, an (oligo)alkylene glycol
35 grouping or another suitable oligomer grouping. Preferred
groupings able to react with amino groups are, for example,

N-hydroxysuccinimide esters, sulfo-N-hydroxysuccinimide esters, imido esters or other activated carboxyl groups; preferred groupings able to react with thiol groups are, for example, maleimide and carboxyl groups; preferred groupings able to react with aldehyde or carboxyl groups are, for example, amino or thiol groups.

5

Examples of linker molecules for connecting SH and NH functions are:

AMAS	(N-α(maleimidoacetoxy)succinimide ester)
BMPS	(N-β(maleimidopropyloxy)succinimide ester)
GMBS	(N-γ(maleimidobutyryloxy)succinimide ester)
EMCS	(N-ε(maleimidocaproyloxy)succinimide ester)
MBS	(m-(maleimidobenzoyl)-N-hydroxysuccinimide ester)
SMCC	(succinimidyl 4-(N-maleimidomethyl)cyclo-hexane-1-carboxylate)
SMPB	(succinimidyl 4-(p-maleimidophenyl)butyrate)
SPDP	(succinimidyl 3-(2-pyridyldithio)proprionate)
Sulfo-GMBS	(N-γ(maleimidobutyryloxy)sulfosuccinimide ester)
Sulfo-EMCS	(N-ε(maleimidocaproyloxy)sulfosuccinimide ester).

10

Examples of linker molecules for connecting SH and SH functions are:

BMB	(1.4-bis-maleimidobutane)
BMDB	(1.4-bis-maleimido-2,3-dihydroxybutane)
BMH	(bis-maleimidohexane)
BMOE	(bis-maleimidoethane)
DTME	(dithio-bis-maleimidoethane)
HBVS	(1.6-hexane-bis-vinyl sulfone)
BM(PEO)$_3$	(1.8-bis-maleimidotriethylene glycol)
BM(PEO)$_4$	(1.11-bis-maleimidotetraethylene glycol).

Examples of linker molecules for connecting NH and NH functions are:

BSOCOES   (bis-(2-succinimidyloxycarbonyloxy)ethyl) sulfone

BS³   (bis-(sulfosuccinimidyl) suberate)

DFDNB   (1.5-difluoro-2,4-nitrobenzene)

DMA   (dimethyl adipimidate HCl))

DSG   (disuccinimidyl glutarate)

DSS   (disuccinimidyl suberate)

EGS   (ethylene glycol bis(succinimidyl succinate).

Examples of linker molecules for connecting SH and CHO functions are:

BMPH   (N-(ß-maleimidopropionic acid)hydrazide TFA)

EMCA   (N-(ε-maleimidocaproic acid)hydrazide)

KMUH   (N-(κ-maleimidoundecanoic acid)hydrazide)

M_2C_2H (4-(N-maleimidomethyl)cyclohexane-1-carboxylhydrazide HCl)

MPBH   (4-(4-N-maleimidophenyl)butyric   acid hydrazide HCl)

PDPH   (3-(2-pyridyldithio)propionylhydrazide).

An example of a linker molecule for connecting SH and OH functions is

PMPI (N-(p-maleimidophenyl) isocyanate).

Examples of linker molecules for converting an SH function into a COOH function are

BMPA   (N-ß-maleimidopropionic acid)

EMCH   (N-ß-maleimidocaproic acid)

KMUA   (N-κ-maleimidoundecanoic acid).

Examples of linker molecules for converting an NH function into a COOH function are MSA (methyl N-succinimidyl adipate) or longer-chain homologues thereof or corresponding derivatives of ethylene glycol.

Examples of linker molecules for converting a COOH function
into an NH function are DAB (1.4-diaminobutane) or longer-
chain homologues thereof or corresponding derivatives of
ethylene glycol.

An example of a linker molecule which reacts with an amino
group of a molecule and provides a protected amino group at
a larger distance from this molecule to avoid steric
hindrance is TFCS (N-ε(trifluoroacetylcaproyloxy)-
succinimide ester).

Further suitable linker molecules are known to skilled
workers and commercially available or can be designed as
required and depending on the functional groups present and
desired in the HAS and the protein to be coupled on, and be
prepared by known methods.

The term "protein" for the purposes of the present
invention is intended to include every amino acid sequence
which comprises at least 9-12 amino acids, preferably at
least 15 amino acids, more preferably at least 25 amino
acids, particularly preferably at least 50 amino acids, and
also include natural derivatives, e.g. pre or proforms,
glycoproteins, phosphoproteins, or synthetic modified
derivatives, e.g. fusion proteins, neoglycoproteins, or
proteins modified by genetic engineering methods, e.g.
fusion proteins, proteins with amino acid exchanges to
introduce preferred coupling sites.

For the prophylactic or therapeutic treatment of the human
or animal body, the relevant protein will carry out a
particular desired function in the body. The protein
therefore preferably has, for example, a regulatory or
catalytic function, a signal transmitting or transport
function or a function in the immune response or induction

of an immune response.

The protein may be selected for example from the group composed of enzymes, antibodies, antigens, transport proteins, bioadhesion proteins, hormones, growth factors, cytokines, receptors, suppressors, activators, inhibitors or a functional derivative or fragment thereof. "Functional derivative or fragment" means in this connection a derivative or fragment which has retained a desired biological property or activity of the parent molecule in whole or in part, e.g. to the extent of at least 10-30%, preferably more than 50%, even more preferably more than 70%, most preferably more than 90%. Particularly preferred examples of such a fragment are antibody fragments.

Specific examples are α-, β- or γ-interferon, interleukins, e.g. IL-1 to IL-18, growth factors, e.g. epidermal growth factor (EGF), platelet growth factor (PDGF), fibroblast growth factor (FGF), brain-derived growth factor (BDGF), nerve growth factor (NGF), B-cell growth factor (BCGF), brain-derived neurotrophic growth factor (BDNF), ciliary neurotrophic factor (CNTF), transforming growth factors, e.g. TGF-α or TGF-β, colony-stimulating factors (CSF), e.g. GM-CSF, G-CSF, BMP (bone morphogenic proteins), growth hormones, e.g. human growth hormone, tumor necrosis factors, e.g. TNF-α or TNF-β, somatostatin, somatotropin, somatomedins, serum proteins, e.g. clotting factors II-XIII, albumin, erythropoietin, myoglobin, hemoglobin, plasminogen activators, e.g. tissue plasminogen activator, hormones or prohormones, e.g. insulin, gonadotropin, melanocyte-stimulating hormone (α-MSH), triptorelin, hypothalamus hormones, e.g. antidiuretic hormones (ADH) and oxytocin, and liberins and statins, parathyroid hormone, thyroid hormones, e.g. thyroxine, thyrotropin, thyroliberin, prolactin, calcitonin, glucagon, glucagon-like peptides (GLP-1, GLP-2, etc.), exendins, e.g.

exendin-4, leptin, vasopressin, gastrin, secretin, integrins, glycoprotein hormones (e.g. LH, FSH, etc.), pigmentary hormones, lipoproteins and apolipoproteins, e.g. Apo-B, Apo-E, Apo-L, immunoglobulins, e.g. IgG, IgE, IgM, IgA, IgD or a fragment thereof, hirudin, tissue pathway inhibitor, plant proteins, e.g. lectin or ricin, bee venom, snake venoms, immunotoxins, antigen E, butroxobina, alpha-proteinase inhibitor, ragweed allergen, melanin, oligolysine proteins, RGD proteins or, where appropriate, corresponding receptors for one of these proteins; or a functional derivative or fragment of one of these proteins or receptors.

Suitable enzymes may be selected for example from the groups of carbohydrate-specific enzymes, proteolytic enzymes, oxidases, oxidoreductases, transferases, hydrolases, lyases, isomerases, kinases and ligases. Specific, non-restrictive examples are asparaginase, arginase, arginine deaminase, adenosine deaminase, glutaminase, glutaminase-asparaginase, phenylalanine ammonia-lyase, tryptophanase, tyrosinase, superoxide dismutase, an endotoxinase, a catalase, peroxidase, kallikrein, trypsin, chymotrypsin, elastase, thermolysin, a lipase, a uricase, adenosine diphosphatase, purine-nucleoside phosphorylase, bilirubin oxidase, a glucose oxidase, glucodase, gluconate oxidase, galactosidase, glucocerebrosidase, glucuronidase, hyaluronidase, tissue factor, a tissue plasminogen activator, streptokinase, urokinase, MAP kinases, DNAses, RNAses, lactoferrin, and functional derivatives or fragments thereof.

As mentioned above, the functional group of the HAS molecule involved in the coupling reaction is the terminal aldehyde group or a group derived therefrom by chemical reaction.

One example of such a chemical reaction is the selective
oxidation of this aldehyde group with a mild oxidizing
agent such as, for example, iodine. bromine or some metal
ions, or else by means of electrochemical oxidation to a
carboxyl group or activated carboxyl group, e.g. an ester,
lactone, amide. with the carboxyl group being converted
where appropriate in a second reaction into the activated
derivative. This carboxyl group or activated carboxyl group
can then be coupled to a primary amino or thiol group of
the protein to form an amide linkage or thioester linkage.

In a particularly preferred preparation method, this
aldehyde group is selectively oxidized with a molar excess
of iodine, preferably in a molar ratio of iodine to HAS of
from 2:1 to 20:1, particularly preferably about 5:1 to 6:1,
in aqueous basic solution. In the optimized method
described in example 1, initially an amount of
hydroxyalkylstarch is dissolved in hot distilled water, and
somewhat less than 1 mole equivalent of aqueous iodine
solution, preferably in a concentration of about 0.05-0.5N,
particularly preferably about 0.1N, is added. After this,
an aqueous NaOH solution in a molar concentration which is
about 5-15 times, preferably about 10 times, that of the
iodine solution is slowly added dropwise, at intervals of a
plurality of minutes, to the reaction solution until the
solution starts to become clear again after the addition.
Somewhat less than 1 mole equivalent of the above aqueous
iodine solution is again added to the reaction solution,
the dropwise addition of the NaOH solution is resumed, and
the addition of iodine and NaOH are repeated until
approximately 5.5-6 mole equivalents of iodine solution and
11-12 mole equivalents of NaOH solution, based on the
hydroxyalkylstarch, have been added. The reaction is then
stopped, the reaction solution is desalted, e.g. by
dialysis or ultrafiltration, subjected to a cation exchange
chromatography, and the reaction product is obtained by

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lyophilization. In this method, virtually quantitative yields are achieved irrespective of the molecular weight of the HAS.

5   In a further particularly preferred embodiment, the selective oxidation takes place with alkaline stabilized solutions of metal ions, e.g. Cu^{++} or Ag^+, likewise in approximately quantitative yields (example 2). It is preferred in this case to employ an approximately 3-10

10   times molar excess of the oxidizing agent.

The selectively oxidized hydroxyalkylstarch (ox-HAS) which has been formed is subsequently reacted in the presence of an activating reagent with a free amino group of the

15   desired protein to form an amide linkage. Examples of suitable activating reagents are N-hydroxysuccinimide, N-hydroxyphthalimide, thiophenol, p-nitrophenol, o,p-dinitrophenol, trichlorophenol, trifluorophenol, pentachlorophenol, pentafluorophenol, 1-hydroxy-1H-

20   benzotriazole (HOBt), HOOBt, HNSA, 2-hydroxypyridine, 3-hydroxypyridine, 3,4-dihydro-4-oxobenzotriazin-3-ol, 4-hydroxy-2,5-diphenyl-3(2H)-thiophenone 1,1-dioxide, 3-phenyl-1-(p-nitrophenyl)-2-pyrazolin-5-one), [1-benzo-triazolyl-N-oxytris(dimethylamino)phosphonium hexafluoro-

25   phosphate] (BOP), [1-benzotriazolyloxytripyrrolidino-phosphonium hexafluorophosphate (PyBOP), [O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), [O-(benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU), [O-(benzotriazol-1-yl)-N,N,N',N'-

30   bis(pentamethylene)uronium hexafluorophosphate, [O-(benzotriazol-1-yl)-N,N,N',N'-bis(tetramethylene)uronium hexafluorophosphate, carbonyldiimidazole (CDI), or preferably carbodiimides, e.g. 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), dicyclohexylcarbodiimide (DCC),

35   diisopropylcarbodiimide (DIPC), particularly preferably EDC. In contrast to conventional methods described in the

literature for similar coupling reactions, it has
surprisingly been found in this connection that on use of a
carbodiimide as a rule the use of otherwise obligatory
further activators such as triazoles, e.g. HOBt, is
5 unnecessary or even makes the yields worse. In the
inventive coupling of ox-HES to various model compounds in
the presence of EDC and absence of HOBt by contrast it was
possible to achieve high yields substantially irrespective
of the molecular weight of the HES (see examples).
10

Instead of the reaction of the carboxyl group or activated
carboxyl group with a free primary amino group of the
protein (e.g. of a lysine or arginine residue), an
analogous reaction with a thiol group (of a cysteine) of
15 the protein is also possible in principle. However, it must
be taken into account in this connection that cysteines are
usually involved in S-S bridges and are therefore not
available for a coupling reaction. If, on the other hand,
free cysteines are present, they frequently play an
20 important part in catalysis or are involved in the contact
site of subunits. A modification of these cysteines will
then result in partial or complete loss of the biological
activity. This problem could be eliminated by introducing
free cysteines by conventional genetic engineering methods
25 such as, for example directed mutagenesis or chemical
peptide synthesis at those sites in the protein which are
known to play no part in the activity. Optimal control of
the coupling site is possible in this way. Targeted
introduction of other reaction amino acids, e.g. Lys, His,
30 Arg, Asp, Glu, into the protein would also be possible in
the same way.

The reactive group of the hydroxyalkylstarch molecule can
also be an amine or thiol group produced by chemical
35 reaction of the terminal aldehyde group. For example, a
reductive amination of the aldehyde group can be carried

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out by reaction with ammonia in the presence of hydrogen and a catalyst or in the presence of sodium cyanoborohydride. The resulting amino or thiol group can then react with a free carboxyl group of the protein (e.g.

5 of an optionally activated glutamic or aspartic acid) to form an amide or thioester linkage.

A further possibility is for the terminal aldehyde group of the hydroxyalkylstarch molecule or a functional group

10 derived therefrom by chemical reaction also to be reacted with a suitable physiologically tolerated bifunctional linker molecule. In this case, the "functional group derived from the terminal aldehyde group of the hydroxyalkylstarch molecule by chemical reaction" for the

15 coupling reaction is the remaining reactive functional group of the bifunctional linker molecule with which the terminal aldehyde group or the functional group derived therefrom has been reacted. It is possible in this way likewise to convert the terminal aldehyde group into a

20 desired functional group.

Suitable linker molecules comprise at one end a group able to enter into a covalent bonding with the terminal aldehyde group or a functional group derived therefrom by chemical

25 reaction, e.g. a carboxyl group, activated carboxyl group, amino or thiol group, and at the other end a group able to enter into a covalent bonding with a reactive functional group of the protein, e.g. an amino, thiol or carboxyl group. Between the two functional groups of the linker

30 molecule there is a biocompatible bridging molecule of suitable length, e.g. a grouping derived from an alkane, an (oligo)alkylene glycol grouping or another suitable oligomer grouping. Preferred groupings able to react with amino groups are, for example, N-hydroxysuccinimide esters,

35 sulfo-N-hydroxysuccinimide esters, imido esters or other activated carboxyl groups; preferred groupings able to

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react with thiol groups are, for example, maleimide and
carboxyl groups; preferred groupings able to react with
aldehyde or carboxyl groups are, for example, amino or
thiol groups.

5

A number of specific, non-restrictive examples of suitable
linker molecules have already been indicated above with
reference to the conjugation of linker molecules to the
protein.

10

In an alternative inventive coupling method of the present
invention, the terminal aldehyde group is reacted directly
with a primary amino group (e.g. of a lysine or arginine
residue or of the N-terminus) of the protein to form a

15 Schiff's base. The formed Schiff's base is, subsequent or
parallel thereto, reduced by reaction with a suitable
reducing agent, resulting in a bonding which is stable in
aqueous medium between protein and HAS. Preferred reducing
agents are sodium borohydride, sodium cyanoborohydride,

20 organic boron complexes, e.g. a 4-(dimethylamino)pyridine-
boron complex, N-ethyldiisopropylamine-boron complex,
N-ethylmorpholine-boron complex, N-methylmorpholine-boron
complex, N-phenylmorpholine-boron complex, lutidine-boron
complex, triethylamine-boron complex, trimethylamine-boron

25 complex; suitable stereoselective reducing agents are, for
example, sodium triacetate borohydride, sodium
triethylborohydride, sodium trimethoxyborohydride,
potassium tri-sec-butylborohydride (K-Selectride), sodium
tri-sec-butylborohydride (N-Selectride), lithium tri-sec-

30 butylborohydride (L-Selectride), potassium
triamylborohydride (KS-Selectride) and lithium triamyl-
borohydride (LS-selectride).

The yields can be improved by suitable variation of the
35 reaction conditions. Parameters for such optimization tests
are the pH of the reaction mixture (possible protein

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degradation by alkaline borohydride), temperature and duration of the incubation, and nature of the reducing agent for the one-pot reaction. A further alternative is the possibility of carrying out the reaction in two steps, in which case an immobilized reducing agent can be employed for the reduction step.

The products of the coupling reaction can be investigated by known methods, and the coupling efficiency can be established. Thus, for example, the free primary amino groups in the protein can be determined before and after the coupling with trinitrobenzenesulfonic acid (Habeeb, ASAF, Anal. Biochem. 14, 328-336 (1966)). The coupling yield of reactions involving primary amines could also be established by derivatization of the unreactive amines with fluorescamine and determination of the fluorescence. The molecular weight distribution can be established by SDS-PAGE and gel permeation. The protein content in the conjugate can be detected by SDS-PAGE and subsequent silver staining, while the saccharide content can be established by a glycan-specific staining of the bands separated by SDS-PAGE after blotting onto a membrane. Quantitative glycan determination is also possible. Exact identification of the coupling site on the protein is possible by peptide mapping and/or MALDI-TOF mass spectroscopy or electrospray ionization mass spectroscopy. It is possible in this way to optimize the coupling and to predetermine the molecular weight distribution and possibly (e.g. if the reactive groups on the protein differ in reactivity) even the coupling site of the products.

The conjugates of the present invention can where appropriate be employed as such or in the form of a pharmaceutical composition for the prophylactic or therapeutic treatment of the human or animal body.

Compositions of this type include a pharmaceutically effective amount of a conjugate of the invention as active ingredient, and a pharmaceutically suitable carrier and, where appropriate, other therapeutic or pharmaceutical

5   ingredients or excipients. Excipients may include for example diluents, buffers, flavorings, binders, surface-active agents, thickeners, lubricants, preservatives (including antioxidants) and substances which serve to make the formulation isotonic with the blood of the intended

10 recipient. A pharmaceutically effective amount is the amount sufficient to display on single or multiple administration a desired beneficial effect during a treatment to alleviate, cure or prevent a pathological condition. A pharmaceutically acceptable carrier is a

15 carrier which is compatible both with the active pharmaceutical ingredient and with the patient's body.

The form of the composition will vary depending on the desired or suitable administration route. A preferred route

20 is parenteral administration, e.g. subcutaneous, intramuscular, intravenous, intraarterial, intraarticular, intrathecal, extradural injection or, where appropriate, infusion. Intranasal, intratracheal or topical administration is also possible. Topical administration of

25 growth factors conjugated according to the invention might for example speed up wound healing. The pharmaceutical compositions may beneficially be supplied in the form of a dosage unit and be produced by any method well known in the pharmacy sector.

30

The conjugates of the present invention can also be employed in all other sectors in which other protein-polymer conjugates, e.g. PEG-protein conjugates, have been used. Some specific, non-restrictive examples are the use

35 of an HAS-protein conjugate as immobilized catalyst or reactant for a reaction in heterogeneous phase or as a

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column material for (immuno)affinity chromatography. Further possible uses will be plainly evident to the skilled worker with knowledge of the properties disclosed herein of the inventive HAS-protein conjugates.

5

The following examples are intended to explain the invention in more detail without, however, restricting it thereto. In particular, analogous reactions can also be carried out with hydroxymethylstarch and hydroxy-propylstarch, and similar results can be achieved.

10

EXAMPLE 1

Selective oxidation of hydroxyethylstarch (HES) with iodine

15   10 g of HES-130 kD were dissolved in 12 ml of deionized water by heating in a round-bottomed flask. 2 ml of an I_2 solution (0.1N) were added to this solution. A pipette with 2 ml of 1.0N NaOH was connected to the flask via a 2-way connector, and the NaOH solution was added dropwise at

20   about 1 drop every 4 minutes. The solution was decolorized after addition of approximately 0.2 ml of the NaOH solution and, at this time, a second portion of 2 ml of 0.1N iodine solution was added. The reaction was complete after addition of a total of 14 ml of iodine solution and 2.8 ml

25   of NaOH solution. The reaction mixture was then dialyzed against deionized water.

Lactonization:

30   The partially desalted solution was subjected to a chromatography on a cation exchange column (Amberlite IR-120, H' form) in order to convert the aldonate groups into aldonic acid groups. Subsequently, the water was removed by lyophilization, and thus the lactone form was

35   obtained.

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Determination of the degree of oxidation:

1 ml of alkaline copper reagent (3.5 g of Na_2PO_4, 4.0 g of K Na tatrate in 50 ml of H_2O, plus 10 ml of 1N NaOH, 8.0 ml
5 of 10% strength (weight/volume) $CuSO_4$ solution and 0.089 g of K iodate in 10 ml of H_2O, after addition of 18 g of Na sulfate, make up to 100 ml) are pipetted in each case into 1 ml of sample solution under an N_2 atmosphere. The mixture is heated at 100°C for 45 minutes. After cooling, 0.2 ml of
10 2.5% strength KI solution and 0.15 ml of 1 M H_2SO_4 are added. After 5 min, 1 drop of phenol red indicator solution (1% weight/volume) is added, and titration is carried out with 5 mM $Na_2S_2O_3$ solution until the color disappears. The concentration of unreacted aldehyde groups can be
15 calculated from the consumption of titrant.

An approximately quantitative yield was achieved (> 98%). It is possible by this procedure to oxidize hydroxyethylstarches with higher molecular weight (e.g.
20 130 kD, 250 kD, 400 kD) just like hydroxyethylstarches with lower molecular weight (e.g. 10 kD, 25 kD, 40 kD), in similarly high yields.

EXAMPLE 2
25 **Selective oxidation of HES with Cu^{2+} ions**

A solution of 0.24 mmol of HES-130 kD was prepared in 10 ml of deionized water with heating. This solution was heated in a 100 ml round-bottomed flask to a temperature of
30 70-80°C, and 1.17 mmol of stabilized Cu^{2+} (e.g. Rochelle salt as stabilizer or other stabilizers) and dilute aqueous NaOH solution was added (final concentration 0.1N NaOH). The temperature was then raised to 100°C, and the reaction was allowed to proceed until a reddish color had appeared.
35 The reaction was stopped and the reaction mixture was cooled to 4°C. The reddish precipitate was removed by

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filtration. The filtrate was dialyzed against deionized water and then converted into the lactone as in example 1 and lyophilized. The oxidation took place quantitatively (yield > 99%). It was also possible by this method to
5 oxidize low molecular weight HES (e.g. HES-10 kD, HES-25 kD, HES-40 kD) and higher molecular weight HES species.

EXAMPLE 3

Coupling of selectively oxidized high molecular weight HES
10 (ox-HES-130 kD) to human serum albumin (HSA)

4.3 g of ox-HES-130 kD and 200 mg of HSA (Sigma, Taufkirchen) were completely dissolved in water by gentle heating in a round-bottom flask with magnetic stirrer.
15 30 mg of ethyldimethylaminopropylcarbodiimide (EDC), dissolved in water, were added to this solution. After stirring very moderately for 2 h, a second portion of 30 mg of EDC was added. After stirring very moderately for a further two hours, a third portion of 40 mg of the
20 carbodiimide was added. The reaction mixture was left under these conditions overnight, dialyzed against distilled water for 15 h and lyophilized. The success of the coupling was demonstrated by gel permeation chromatography, SDS-PAGE and carbohydrate-specific staining (Glyco-Dig kit from
25 Roche-Boehringer, Basle) after blotting onto a PVDF membrane. The yield of coupling product was about 90%.

EXAMPLE 4

Coupling of selectively oxidized low molecular weight HES
30 (ox-HES-10 kD) to human serum albumin (HSA)

7.4 g of ox-HES-10 kD and 50 mg of HSA were completely dissolved in water in a round-bottom flask with magnetic stirrer. The reaction was carried out by the method
35 described above for high molecular weight HES, adding a total of 282 mg of EDC in three aliquots. The reaction

mixture was likewise dialyzed and lyophilized as described
above. Analysis (as above) showed the coupling product was
obtained, but the yields were somewhat lower than in the
coupling with high molecular weight ox-HES.

5

EXAMPLE 5
Coupling of ox-HES-130 kD to myoglobin (Mb)

4.3 g of ox-HES-130 kD were completely dissolved in water
10   (6-7 ml), and then 100 mg of Mb (Sigma, Taufkirchen),
dissolved in 10 ml of 0.1 M phosphate buffer (pH 7.0), were
added. The coupling reaction was started by adding 30 mg of
EDC. Addition of EDC was repeated every 2 hours until a
total of 90 mg of the carbodiimide had been consumed. The
15   reaction mixture was then dialyzed against 50 mM phosphate
buffer, pH 7.0, and lyophilized. GPC showed a definite
product peak, which was detected in the hold-up volume at
450 nm. It was possible to calculate a coupling yield of
88% from this. The oxygen-binding capacity of the hesylated
20   myoglobin was about 76% of the binding capacity of
unmodified Mb.

EXAMPLE 6
Coupling of ox-HES-10 kD to superoxide dismutase (SOD)

25

One part by volume of an aqueous solution of ox-HES-10 kD
(1.05 g/ml) was incubated with one part by volume of a
7 mg/ml SOD solution (Sigma, Taufkirchen) in 50 mM
phosphate buffer, pH 7.6, at room temperature. The coupling
30   reaction was initiated by adding 280 mg of EDC in
5 portions over a period of 24 h. The progress of the
reaction was followed by GPC analysis in phosphate buffer
and detection at 280 nm. After 24 h, 81% of the protein
were found in the higher molecular weight region of the
35   separating column, and the reaction was stopped after this
time. The reaction mixture was subjected to a diafiltration

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with a 30 kD membrane and then lyophilized. Mass spectrometric analysis of the product showed an average molar ratio of HES to protein of about 3:1.

5 EXAMPLE 7

Coupling of ox-HES-130 kD to streptokinase (SK)

3.8 kg of ox-HES-130 kD were dissolved together with 35 mg of streptokinase (Sigma, Taufkirchen) in the minimum amount
10 of 50 mM phosphate buffer, pH 7.2. At room temperature, 46.5 mg of EDC and 20 mg of 1-hydroxybenzotriazole hydrate (HOBt) were added, and reaction was maintained with gentle stirring for a total of 24 h. After dialysis and freeze drying, about 79% of the protein were found as HES
15 conjugate by GPC analysis. In the SDS-PAGE with silver staining, a distinct increase in the molecular mass of the streptokinase was observable. In parallel with this, carbohydrate structures were unambiguously detectable in the high molecular waveband with the digoxigenin method.
20

EXAMPLE 8

Coupling of ox-HES-130 kD to human interleukin-1 (IL-2)

45 mg of ox-HES-130 kD were completely dissolved in 0.5 ml
25 of 50 mM Na phosphate buffer, pH 6.5, with gentle heating. After addition of 0.25 mg of human IL-2 (Sigma, Taufkirchen), which made the solution opaque, the mixture was stirred at room temperature for 4-6 h. Then 5 mg of EDC were added in 4 portions with a time difference of 2 h for
30 each, and stirring was continued overnight, resulting in a clear solution. GPC analysis revealed a coupling yield of about 65%.

EXAMPLE 9

Coupling of ox-HES-25 kD to human tumor necrosis factor α (TNFα)

5   0.3 mg of hTNFα (Sigma, Taufkirchen) were added to 86 mg of ox-HES-25 kD in about 0.4 ml of 0.1 M phosphate buffer (pH 7.0). The cloudy solution was stirred for about 2 h before 1 mg of EDC and 0.5 mg of HOBt were added. Stirring was continued for about 6 h, with the solution becoming clear
10   during the reaction time. The coupling product was isolated by ultrafiltration and freeze drying and analyzed by GPC and detection at 280 nm. A coupling yield of approximately 74% was found in this case.

15   **EXAMPLE 10**

Coupling of ox-HES-130 kD to glucagon-like peptide (GLP-1)

7.4 g of ox-HES-130 kD were dissolved in a minimum volume of water by heating and gentle stirring. A solution of
20   10 mg of GLP-1 in the amide form (Bachem, Switzerland) in 50 mM phosphate buffer, pH 7.4, was added by pipette. The reaction was started by adding 35 mg of EDC and was cautiously stirred for 2 h. This was repeated 2x more because, after this time, a peptide peak was no longer
25   evident in the GPC analysis at 280 nm, i.e. approximately complete conversion to the coupling product had taken place. This coupling product was diafiltered using a 30 kD membrane and lyophilized from phosphate buffer solution. It was possible to conclude from the results of a MALDI mass
30   spectroscopy that the stoichiometry between peptide and HES was 1:1.

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EXAMPLE 11

Coupling of high molecular weight HES (HES-130 kD) to human serum albumin (HSA)

5   9.75 g of HES-130 KD were completely dissolved in water (6-7 ml), and then 50 mg of HSA, dissolved in 1 ml of 0.1 M phosphate buffer (pH 7.4) were added. The reaction mixture was stirred with a magnetic stirrer. The solution was then mixed with NaBH₃CN (50-70 mg) and stirred gently for a few

10 minutes. The solution was further stirred for 15 minutes every two hours. Then a further aliquot of NaBH₃CN (about 50 mg) was added. At the end (after a reaction time of almost 36 h), a total amount of 285 mg of NaBH₃CN had been employed. The solution was then dialyzed and lyophilized.

15 Analysis took place as described in example 4. The coupling efficiency was about 65%.

EXAMPLE 12

Coupling of low molecular weight HES (HES-130 kD) to human
20 serum albumin (HSA)

4.5 g of HES were completely dissolved in water (4-5 ml) and 50 mg of HSA, dissolved in 1 ml of 0.1 M phosphate buffer (pH 7.4) were added. When the solution was clear, if
25 necessary effected by stirring with a magnetic stirrer, NaBH₄ (50-70 mg) was added and mixed in with gentle stirring. The solution was left to stand without stirring for two hours and then stirred for 15 minutes every two hours as for the reaction with high molecular weight HES.
30 When the solution no longer showed any bubbles (H₂ evolution), a further aliquot of NaBH₄ (about 50 mg) was added. At the end, a total amount of 180 mg of NaBH₄ had been employed. The solution was then dialyzed and lyophilized. Analysis took place by gel permeation
35 chromatography (GPC), and the yield was about 15%.

EXAMPLE 13
Coupling of HES-40 kD to asparaginase

3.0 g of HES-40 kD were completely dissolved in water
5 (about 4 ml). A solution of 80 mg of asparaginase (Sigma,
Taufkirchen) in 6 ml of 0.1 M borate buffer, pH 9.0, were
added thereto and stirred until the reaction mixture was
clear. The temperature was then raised to 37°C and, after
2 h, about 50 mg of NaBH₃CN were added. This reaction cycle
10 was repeated 3x more. The product was worked up by
dialyzing the reaction mixture against 0.1 M phosphate
buffer, pH 7.4. The yield of coupling product was about
61%, and about 73% of the asparaginase activity was
recoverable.
15

EXAMPLE 14
Coupling of HES-130 kD to human interleukin-2 (IL-2)

50 mg of HES-130 kD were completely dissolved in water
20 (about 0.2 ml). A suspension of 0.25 mg of human IL-2
(Sigma, Taufkirchen) in 0.2 ml of 0.1 M borate buffer, pH
9.0, was added thereto and stirred until the reaction
mixture was clear (4 h). 1 mg portions of NaBH₄CN were
added at intervals each of 4 h, and stirring was continued.
25 After a further reaction time of 24 h, the mixture was
dialyzed against 0.1 M phosphate buffer, pH 7.4 and
lyophilized. The yield of coupling product was about 42%
according to GPC analysis.

30 ## EXAMPLE 15
Coupling of HES-130 kD to insulin

4.0 g of HES-130 kD were completely dissolved in water
(about 6 ml). 55 mg of insulin from bovine pancreas (Sigma,
35 Taufkirchen) in 7.5 ml of 0.1 M borate buffer (pH 9.0),
were added thereto and stirred at 37°C for about 24 h. The

reducing agent NaBH$_3$CN (60 mg in 30 ml) was slowly added dropwise over a period of 8 h. The reaction mixture was then stirred for a further 24 h and freed of faults and unreacted reagents by ultrafiltration (30 kD).
5 Lyophilization resulted in a stable coupling product. About 95% of the insulin employed was recovered as HES conjugate.

EXAMPLE 16

Coupling of ox-HES-130 kD to superoxide dismutase (SOD)
10

130 mg of ox-HES-130 kD were completely dissolved in 6 ml of PBS pH 6, and then 10 mg of SOD (Roche, Mannheim) dissolved in 1 ml of PBS pH 6 were added. The coupling reaction was started by adding 10 mg of EDC. Addition of
15 EDC was repeated every 3 h until 39 mg of the carbodiimide had been consumed. The reaction was monitored by GPC at 258 nm. After 24 h, 50% of the protein were found in the high molecular weight region of the separating column, and the reaction was stopped. The reaction mixture was dialyzed
20 against 25 mM phosphate buffer pH 7.2 and lyophilized. The SOD activity was 95% of the initial activity. Determination of the mass distribution of HES protein samples by coupled GPC-light scattering revealed a molar ratio of HES to protein of 1:1.
25

EXAMPLE 17

Coupling of ox-HES 70 kD to glucagon

Glucagon (66 × 10^{-9} mol, 0.23 mg), oxHES 70 kD
30 (6.6 × 10^{-6} mol, 123 mg) were dissolved in phosphate buffer (1 ml, pH 5) in a round-bottom flask. 26 mg of EDC were added in 10 portions at intervals of 1 h. After a reaction time of 24 h, the reaction was stopped by adding 10 ml of water. The coupling product was purified by after dialysis
35 against water by GPC and ion exchange chromatography. Freeze drying resulted in 88 mg of white coupling product (73%).

CLAIMS

1. A hydroxyalkylstarch-protein conjugate, characterized
   in that the binding interaction between the
   hydroxyalkylstarch molecule and the protein is based on
   a covalent bonding which is the result of a coupling
   reaction between (i) the terminal aldehyde group, or a
   functional group derived from this aldehyde group by
   chemical reaction, of the hydroxyalkylstarch molecule
   and (ii) a functional group, which is able to react
   with this aldehyde group or functional group derived
   therefrom of the hydroxyalkylstarch molecule, of the
   protein, where the bonding resulting directly in the
   coupling reaction can be modified where appropriate by
   a further reaction to give the abovementioned covalent
   bonding.

2. The hydroxyalkylstarch-protein conjugate as claimed in
   claim 1, characterized in that the functional group
   derived from the terminal aldehyde group of the
   hydroxyalkylstarch molecule by chemical reaction is one
   of the functional groups of a bifunctional linker
   molecule with which the terminal aldehyde group has
   been reacted.

3. The hydroxyalkylstarch-protein conjugate as claimed in
   claim 1 or 2, characterized in that the reactive
   functional group of the protein is one of the
   functional groups of a bifunctional linker molecule
   which has been coupled onto the protein.

4. The hydroxyalkylstarch-protein conjugate as claimed in
   claim 1 or 2, characterized in that the reactive
   functional group of the protein has been introduced
   into the protein by recombinant modification of the
   original amino acid sequence.

5. The hydroxyalkylstarch-protein conjugate as claimed in claim 1, 3 or 4, characterized in that the covalent bonding is the result of a coupling reaction between a carboxyl group formed by selective oxidation of the terminal aldehyde group, or activated carboxyl group, of the hydroxyalkylstarch molecule and a primary amino group or thiol group of the protein.

6. The conjugate as claimed in claim 5, characterized in that the covalent bonding is an amide linkage which is the result of a coupling reaction between an activated carboxyl group formed by selective oxidation of the terminal aldehyde group of the hydroxyalkylstarch molecule, and a primary amino group of the protein.

7. The conjugate as claimed in claim 1, 3 or 4, characterized in that the covalent bonding is an amine linkage which is the result of a coupling reaction between the terminal aldehyde group of the hydroxyalkylstarch molecule and a primary amino group of the protein to form a Schiff's base, and reduction of the Schiff's base to the amine.

8. The conjugate as claimed in any of claims 1 to 7, characterized in that the hydroxyalkylstarch molecule has a molecular weight in the range from about 4 to about 1000 kD.

9. The conjugate as claimed in claim 8, characterized in that the hydroxyalkylstarch molecule has a molecular weight of about 4 to about 50 kD.

10. The conjugate as claimed in claim 8, characterized in that the hydroxyalkylstarch molecule has a molecular weight of about 70 to about 1000 kD.

11. The conjugate as claimed in claim 10, characterized in that the hydroxyalkylstarch molecule has a molecular weight of about 130 kD.

12. The conjugate as claimed in any of claims 1 to 11, characterized in that the hydroxyalkylstarch molecule has a degree of substitution of about 0.3 to about 0.7.

13. The conjugate as claimed in any of claims 1 to 12, characterized in that the hydroxyalkylstarch molecule has a ratio of C_2 to C_6 substitution of from 8 to 12.

14. The conjugate as claimed in any of claims 1 to 13, characterized in that the hydroxyalkylstarch molecule is a hydroxyethylstarch molecule.

15. The conjugate as claimed in any of claims 1 to 14, characterized in that the protein has a regulatory or catalytic function, a signal transmitting or transport function or a function in the immune response or induction of an immune response.

16. The conjugate as claimed in claim 15, characterized in that the protein is selected from the group composed of enzymes, antibodies, antigens, transport proteins, bioadhesion proteins, hormones and prohormones, growth factors and growth factor receptors, cytokines, receptors, suppressors, activators, inhibitors or a functional derivative or fragment thereof.

17. The conjugate as claimed in claim 15 or 16, characterized in that the protein is α-, β- or γ-interferon, an interleukin, a serum protein, e.g. albumin or a clotting factor, erythropoietin, myoglobin, hemoglobin, a plasminogen activator, BCGF,

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BDGF, EGF, FGF, NGF, PDGF, BDNF, CNTF, TGF-α, TGF-β, a colony-stimulating factor, a BMP, somatomedin, somatotropin, somatostatin, insulin, gonadotropin, α-MSH, triptorelin, prolactin, calcitonin, glucagon, a glucagon-like peptide, e.g. GLP-1 or GLP-2, exendin, leptin, gastrin, secretin, an integrin, a hypothalamus hormone, e.g. an ADH, oxytocin, a liberin or statin, a thyroid hormone, e.g. thyroxine, thyrotropin, thyroliberin, a growth hormone, e.g. human growth hormone, LH, FSH, a pigmentary hormone, TNF-α or TNF-β, hirudin, a lipoprotein or apolipoprotein, e.g. Apo-B, Apo-E, Apo-Lp, an oligolysine protein, an RGD protein, a lectin or ricin, bee venom or a snake venom, an immunotoxin, ragweed allergen, antigen E, an immunoglobulin, or a receptor for one of these proteins or a functional derivative or fragment of one of these proteins or receptors.

18. The conjugate as claimed in claim 15 or 16, characterized in that the protein is an enzyme which is selected from an asparaginase, arginase, arginine deaminase, adenosine deaminase, glutaminase, glutaminase-asparaginase, phenylalanine ammonia-lyase, tryptophanase, tyrosinase, superoxide dismutase, endotoxinase, catalase, peroxidase, kallikrein, trypsin, chymotrypsin, elastase, thermolysin, a lipase, uricase, adenosine diphosphatase, purine-nucleoside phosphorylase, bilirubin oxidase, glucose oxidase, glucodase, gluconate oxidase, galactosidase, glucocerebrosidase, glucuronidase, hyaluronidase, tissue factor, a tissue plasminogen activator, streptokinase, urokinase, an MAP kinase, DNAse, RNAse, lactoferrin, and functional derivatives or fragments thereof.

19. A pharmaceutical composition comprising an effective

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amount of a conjugate as claimed in any of claims 1 to
18 and a pharmaceutically acceptable carrier and, where
appropriate, further excipients and active ingredients.

5   20. The use of a conjugate as claimed in any of claims 1 to
18 or a composition as claimed in claim 19 for the
therapeutic or preventive treatment of humans or
animals.

10   21. A method for preparing a hydroxyalkylstarch-protein
conjugate as claimed in any of claims 1 to 18,
characterized in that a coupling reaction is carried
out in aqueous solution between the terminal aldehyde
group, or a functional group derived from this aldehyde
15   group by chemical reaction, of the hydroxyalkylstarch
molecule and a functional group, which is able to react
with this aldehyde group or functional group derived
therefrom of the hydroxyalkylstarch molecule, of the
protein, and the bonding resulting directly in the
20   coupling reaction is modified where appropriate by a
further reaction.

22. The method as claimed in claim 21, characterized in
that the reaction medium of the coupling reaction is
25   water or a mixture of water and an organic solvent,
where the water content of the mixture is at least 80%.

23. The method as claimed in claim 21 or 22, characterized
in that the terminal aldehyde group of the
30   hydroxyalkylstarch molecule is converted by selective
oxidation into the corresponding carboxyl
functionality, and the latter is subsequently reacted
under activating conditions in aqueous solution with a
free amino group of the protein, so that the
35   hydroxyalkylstarch molecule is linked to the protein by
an amide linkage.

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24. The method as claimed in claim 23, characterized in that the selective oxidation of the aldehyde group is carried out with iodine or metal ions in basic aqueous solution.

25. The method as claimed in claim 23 or 24, characterized in that the coupling reaction is carried out in the presence of a carbodiimide.

26. The method as claimed in claim 25, characterized in that the carbodiimide is 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC).

27. The method as claimed in claim 21 or 22, characterized in that the terminal aldehyde group of the hydroxyalkylstarch molecule is coupled to a free amino group of the protein to form a Schiff's base, and the formed Schiff's base is reduced to the amine, so that the hydroxyalkylstarch molecule is linked to the protein by an amine linkage.

28. The method as claimed in claim 27, characterized in that both coupling and reduction take place in aqueous solution.

29. The method as claimed in claim 27 or 28, characterized in that the reducing agent is sodium borohydride, sodium cyanoborohydride or an organic boron complex.

30. The method as claimed in any of claims 27 to 29, characterized in that the coupling and reduction reactions are carried out simultaneously.

31. A method for preparing hydroxyalkylstarch which is selectively oxidized at the terminal aldehyde group,

characterized in that the hydroxyalkylstarch is reacted in a molar ratio of iodine to HAS of from 2:1 to 20:1 in basic aqueous solution.

32. The method as claimed in claim 31, characterized in that the molar ratio of iodine to HAS is about 5:1 to 6:1.

33. The method as claimed in claim 31, characterized in that

a) an amount of hydroxyalkylstarch is dissolved in warm distilled water, and somewhat less than 1 mole equivalent of aqueous iodine solution is added,

b) NaOH solution in a molar concentration which is about 5-15 times that of the iodine solution is slowly added dropwise, at intervals of a plurality of minutes, to the reaction solution until the solution starts to become clear again after the addition,

c) somewhat less than 1 mole equivalent of aqueous iodine solution is again added to the reaction solution,

d) the dropwise addition of the NaOH solution is resumed,

e) steps b) to d) are repeated until approximately 5.5-6 mole equivalents of iodine solution and 11-12 mole equivalents of NaOH solution, based on the hydroxyalkylstarch, have been added,

f) the reaction is then stopped, and the reaction solution is desalted and subjected to a cation exchange chromatography, and the reaction product is obtained by lyophilization.

34. The method as claimed in claim 33, characterized in that the aqueous iodine solution is an approximately 0.05-0.5N iodine solution.

35. The method as claimed in claim 33 or 34, characterized in that the molar concentration of the NaOH solution is about 10 times that of the iodine solution.

36. A method for preparing hydroxyalkylstarch which is selectively oxidized at the terminal aldehyde group, characterized in that the HAS is oxidized in aqueous alkaline solution with a molar excess of stabilized metal ions selected from Cu^{2+} ions and Ag^+ ions.